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Immunosuppressive Effects of Fisetin in Ovalbumin-Induced Asthma through Inhibition of NF-κB Activity

Mei-Yao Wu,[†] Shih-Kai Hung,[†,‡,§] and Shu-Ling Fu*[,†,∥]

[†]Institute of Traditional Medicine, National Yang-Ming University, Taipei, Taiwan, 11221

[‡]Department of Radiation Oncology, Buddhist Dalin Tzu Chi General Hospital, Chiayi, Taiwan, 62247

[§]School of Medicine, Tzu Chi University, Hualian, Taiwan, 97061

[∥]Molecular Medicine Program, National Yang-Ming University, Taipei, Taiwan, 11221

ABSTRACT: Fisetin, a flavonoid compound commonly present in fruits and vegetables, can exert anti-inflammation activities via inhibition of the NF-κB-signaling pathway. This study aims to evaluate the antiasthma activity of fisetin and investigate its possible molecular mechanisms. We found that fisetin attenuated lung inflammation, goblet cell hyperplasia, and airway hyperresponsiveness in ovalbumin-induced asthma and decreased eosinophils and lymphocytes in bronchoalveolar lavage fluid. Fisetin treatment reduced expression of the key initiators of allergic airway inflammation (eotaxin-1 and TSLP), Th2-associated cytokines (IL-4, IL-5, and IL-13) in lungs, and Th2-predominant transcription factor GATA-3 and cytokines in thoracic lymph node cells and splenocytes. Notably, fisetin treatment impaired NF-κB activation in OVA-stimulated lung tissues and TNF-α-stimulated bronchial epithelial cells. Collectively, this study demonstrated the beneficial effect of fisetin in the amelioration of asthmatic phenotypes. The antiasthma activity of fisetin is associated with reduction of Th2 responses as well as suppression of NF-κB and its downstream chemokines.

KEYWORDS: Asthma, fisetin, NF-κB

INTRODUCTION

Fisetin (3,7,3′,4′-tetrahydroxyflavone, the structure was reported previously[1]) is commonly found in fruits and vegetables such as strawberries, grapes, and onions.[2] Fisetin has multiple pharmacological effects including antioxidation,[3] anticarcinogenesis,[4] antiangiogenesis,[5] and immunosuppression.[1,6,7] Particularly, the anti-inflammatory effects of fisetin have been demonstrated both in vitro and in vivo. Fisetin has been shown in vitro to suppress the secretion of IL-4, IL-5, and IL-13 by activated basophils,[6] inhibit histamine release and cytokine expression (IL-4 and TNF-α) in human mast cells,[7,8] suppress the activation of macrophages and peripheral blood mononuclear cells (PBMC), and impair maturation of dendritic cells.[1,9,10] Fisetin was found to inhibit NF-κB activation via suppressing its upstream signaling molecules including TAK-1 and IκB-α kinase (IKK) in cancer cells.[4] Animal studies revealed that both LPS-induced acute pulmonary inflammation and collagen-induced arthritis were attenuated by fisetin.[11,12] Jung et al. demonstrated that flavonols attenuated the acute asthmatic responses through inhibition of eosinophils and neutrophils in the conscious guinea pigs.[13]

Allergic asthma (a chronic disease of the bronchial epithelium, mucus-secreting glands, and lung parenchyma) is characterized by lung inflammation, airway hyperresponsiveness (AHR), and mucus overproduction.[14] Evidence from clinical and preclinical studies reveals the immunopathogenesis of allergic asthma involving aberrant activation of pro-inflammatory cells, T helper 2 (Th2) cells, eosinophils, mast cells, and B cells and associated changes in levels of cytokines (IL-4, IL-5, and IL-13).[15] Activation and survival of eosinophils, the hallmark of allergic asthma, are mainly related to IL-5,[16] and the recruitment of circulating eosinophils into lungs is driven by eotaxin-1 (also known as chemokine ligand 11, CCL11) and IL-13.[17] AHR and mucus production are also induced by IL-13.[18] It was shown that IL-4 and IL-13 can activate B cells for IgE production and thereby activate mast cells and histamine release.[14]

Although Th2 cells and eosinophils have been considered the main cell types involved in the pathogenesis of asthma, emerging evidence has revealed the important contribution of bronchial epithelial cells as the first line barrier to aspirated allergens.[14] Upon stimulation by antigen or proinflammatory cytokines, epithelial cells secrete various mediators including thymic stromal lymphopoietin (TSLP), a key initiator of allergic airway inflammation. TSLP activates asthma-related inflammatory cells including eosinophils, basophils, dendritic cells, and Th2 cells,[19] and its transcription is triggered by nuclear factor-κB (NF-κB).[20] Indeed, the pivotal importance of NF-κB activation in bronchial epithelial cells and immune cells has been demonstrated both in murine models of asthma and in asthma patients.[21] Therefore, NF-κB has emerged as a promising molecular target for the treatment of asthma.[22]

To further explore the immunopharmacological potential of fisetin, we herein evaluated whether fisetin could attenuate OVA-induced asthmatic phenotypes and investigated molecular mechanisms underlying fisetin-mediated antiasthma activity.

MATERIALS AND METHODS

Chemicals and Antibodies. Fisetin, chicken ovalbumin (OVA, grade V), and methacholine were purchased from Sigma-Aldrich

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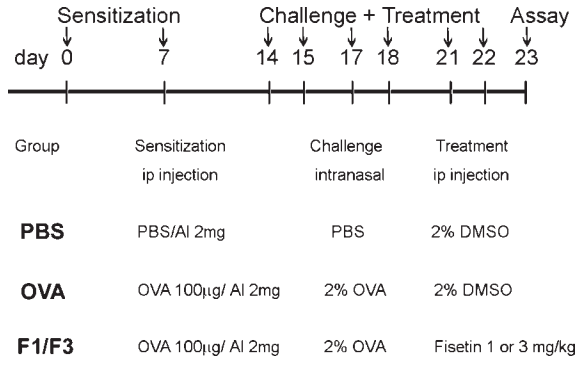

Figure 1. Scheme for OVA induction of asthma in mice and fisetin treatment. The protocol of sensitization, challenge, and treatment was described in the Materials and Methods. PBS group, PBS challenge with DMSO treatment; OVA group, OVA challenge with DMSO treatment; and F1/F3 groups, OVA challenge with fisetin treatment (1 or 3 mg/kg). The same designation for each treatment group is used in all subsequent figures.

(St. Louis, MO). $Al(OH)_3$ was from Pierce Biotechnology (Rockford, IL). Antibodies used in this study include antiphospho-IκB-α (Ser 32) and anti-IκB-α (Cell Signaling, Danvers, MA), anti-p65 (Abcam, Cambridge, United Kingdom), anti-GAPDH, and anti-Histone H3 (GeneTex, Irvine, CA).

Cell Culture. The immortalized human bronchial epithelial cell line, NL 20, was purchased from Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan) and cultured in Ham's F12 medium supplemented with 2 mM L-glutamine, 0.1 mM nonessential amino acids, 0.005 mg/mL insulin, 10 ng/mL epidermal growth factor, 0.001 mg/mL transferrin, 500 ng/mL hydrocortisone, and 4% fetal bovine serum.

OVA-Induced Murine Asthma Model and Fisetin Treatment Protocol. The experimental protocol is shown in Figure 1. Male BALB/c mice (6−8 weeks old) purchased from National Laboratory Animal Center were sensitized with 100 μg of OVA plus 2 mg of $Al(OH)_3$ in 100 μL of phosphate-buffered saline (PBS) through intraperitoneal (ip) injection on day 0 and day 7. The sensitized mice were then challenged with aerosolized 2% OVA in PBS for 30 min on days 14, 15, 17, 18, 21, and 22. Fisetin [1 or 3 mg/kg, in 2% dimethyl sulfoxide (DMSO)] or vehicle (2% DMSO in PBS) was given through ip injection at 12 and 1 h before each OVA challenge. Animals sensitized with PBS containing $Al(OH)_3$ and challenged with aerosolized PBS were used as the negative controls. The animal study was performed according to the Institutional Guidelines for Animal Care and Use Committee at the National Yang Ming University (approval number: 1000311).

Bronchoalveolar Lavage (BAL) Fluid Analysis. Mice were anesthetized by pentobarbital (60 mg/kg, ip) 24 h after the final aerosol challenge. BAL was performed as described previously.[23] Cells from BAL fluid were suspended in PBS and counted, and cytospins were prepared (200 rpm, 10 min) and stained with Wright-Giemsa. Differential counts of at least 300 cells were carried out in the high power field of a microscope, and cells were identified based on their morphological features. Moreover, the BAL fluid was collected to analyze cytokine levels using enzyme-linked immunosorbent assay (ELISA).

Histological Analysis. Lung tissues were fixed with 10% formalin, embedded in paraffin, and cut into 3 μm sections, which were stained with hematoxylin and eosin (H&E stain) to determine lung inflammation and cell infiltration. The sections were also stained with periodic acid-Schiff (PAS) and counterstained with hematoxylin to examine goblet cell hyperplasia.

Measurement of AHR. Twenty-four hours after the final aerosol challenge, AHR was measured in unrestrained mice using a whole body plethysmograph (Buxco Electronics, Troy, NY). Before recording, the chambers were calibrated with an injection of 1 mL of air. Conscious mice received aerosol challenge with methacholine at increasing concentrations (0−20 mg/mL in saline) for 3 min. Enhanced pause (Penh) was recorded for 3 min after each challenge. Penh = pause × PIF/PEF; pause = (Te − Rt)/Rt (PIF, peak inspiratory height; PEF, peak expiratory height; Te, expiratory time; and Rt, time to expire 65% of the volume).

Table 1. List of Primers Used for Real-Time PCR

name	sequence
GATA3-F (mouse)	5′-CGAGACATAGAGAGCTACGCAATCT-3′
GATA3-R (mouse)	5′-CCTGAGTAGCAAGGAGCGTAGAG-3′
eotaxin-1-F (mouse)	5′-GAGCCTAAGAACTGCTTGATTCCT-3′
eotaxin-1-R (mouse)	5′-GGGCGACTGGTGCTGATATT-3′
TSLP-F (mouse)	5′-ATCCAGCTGTCAAAGGGTTCA-3′
TSLP-R (mouse)	5′-TCTCGTAGATGTGCAAGGCTCTT-3′
IL-13-F (mouse)	5′-CCTGGATTCCCTGACCAACA-3′
IL-13-R (mouse)	5′-TTACAGAGGCCATGCAATATCCT-3′
GAPDH-F (mouse)	5′-TGTGATGGGTGTGAACCACGA-3′
GAPDH-R (mouse)	5′-TGCTGTTGAAGTCGCAGGAGAC-3′

Isolation of Thoracic Lymph Node (TLN) Cells and Splenocytes. TLN cells and splenocytes were used to determine the immune-regulatory effects of fisetin. After the asthmatic mice were sacrificed, TLNs and spleens were isolated, and cell clumps were disaggregated into single cell suspensions using filtration through nylon mesh (30 μm). Red blood cells were lysed by addition of lysis buffer. The isolated TLN cells and splenocytes were cultured at the density of 3×10^6/mL in 24-well plates under the stimulation with 200 μg/mL OVA for 96 h. The culture medium was collected to detect cytokine levels by ELISA, and the expression of immune-regulatory genes in these cells was determined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR).

Measurement of Gene Expression by qRT-PCR. Total RNA was extracted from lung tissues or cultured cells with Trizol reagent according to the manufacturer's instructions (Sigma-Aldrich), and the concentration of RNA was determined. cDNA was synthesized from 2 μg of total RNA using the RevertAid First Strand cDNA Synthesis kit (Fermentas, Burlington, Canada). The cDNA product from each sample was analyzed by real-time PCR using the StepOnePlus Real-Time PCR system (Applied Biosystems). The primers were designed using Primer Express 2.0 (Applied Biosystems, Foster City, CA), and their sequences are listed in Table 1. GAPDH serves as the internal control. The relative expression of the target gene versus GAPDH was determined using "$2^{-\Delta CT}$" where ΔCT represents the CT (threshold cycle) value of the target gene minus the CT value of GAPDH.

Cytokine Assay. Lung tissues were homogenized with 1 mL of protein extraction buffer (50 mM Tris-HCl, pH 7.4, 15 mM NaOH, 0.1% NP-40, 5 mM EDTA, and 10 mM EGTA) containing protease inhibitor (Sigma-Aldrich), and the supernatant was collected after centrifugation. The levels of IL-4, IL-5, and IFN-γ in the supernatant of the homogenized lung tissues, BAL fluid, and media from cultured TLN cells and splenocytes were all measured by ELISA (R&D Systems, Bloomington, MN). The detection limit was 7.8 pg/mL.

Luciferase Assay. NL 20 cells were seeded at the density of 5×10^4/ml in 24-well plates, incubated for 24 h, and transfected with 0.5 μg of luciferase reporter plasmid containing NF-κB binding sites (NF-κB-Luc, Stratagene, La Jolla, CA) and 0.05 μg of β-galactosidase plasmid using TurboFect reagent (Fermentas). Twenty-four hours later, the cells were pretreated with vehicle (0.1% DMSO) or fisetin (1, 3, and 10 μM) for 1 h, stimulated with 10 ng/mL of TNF-α for 5 h, lysed in 100 μL of lysis buffer (Promega, Madison, WI), and assayed for luciferase activity

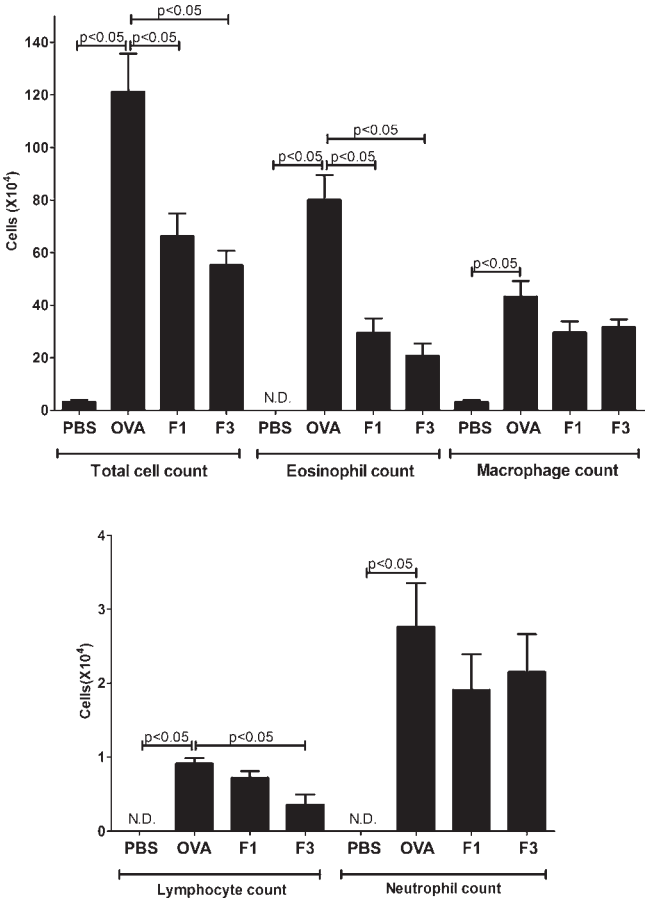

Figure 2. Fisetin suppressed accumulation of inflammatory leukocytes in the airways of OVA-challenged mice. Total cell counts and differential counts of at least 300 cells in BAL fluid from mice subjected to various treatments were determined 24 h after the final challenge. PBS, $n = 5$; OVA, $n = 9$; F1, $n = 6$; and F3, $n = 6$. Values are shown as means ± SEMs, and $p < 0.05$ indicates significant difference.

(Promega) as described previously.[24] Luciferase activities were normalized to β-galactosidase activities.

Measurement of Protein Expression. Lung tissues were homogenized in liquid nitrogen. Cytosolic fractions were extracted using cytosolic lysis buffer (10 mM Tris, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% NP-40), and nuclear fractions were extracted using nuclear lysis buffer (20 mM HEPES, pH 7.9, 25% glycerol, 0.4 M NaCl, and 1.5 mM $MgCl_2$). Cytosolic and nuclear protein lysates (50 μg per lane) were separated on 10% SDS-PAGE and immunoblotted as previously described.[24] The PVDF membranes were probed with antiphospho-IκB-α, anti-IκB-α, anti-p65, anti-histone H3, and anti-GAPDH antibodies. The intensity of image was quantified using Image J program version 1.40 (NIH, Bethesda, MD).

Statistical Analysis. Data are presented as means ± standard errors of the mean (SEMs). One-way analysis of variance followed by the Dunnett test was used to determine the significance of between-group differences. Statistical significance was set at $p < 0.05$.

RESULTS

Fisetin Inhibited OVA-Induced Inflammatory Cell Infiltration and Goblet Cell Hyperplasia. The experimental protocols for establishing the OVA-induced asthma model and treatment with fisetin are outlined in Figure 1. To investigate whether fisetin could suppress the OVA-induced infiltration of lungs by inflammatory cells, we performed BAL 24 h after the final aerosol challenge and examined each specimen using Wright-Giemsa stain. OVA challenge significantly increased the total number of cells in BAL fluid, as well as the numbers of eosinophils, macrophages, lymphocytes, and neutrophils (Figure 2). Fisetin (as compared with vehicle alone) significantly decreased the total number of cells and number of eosinophils but not the number of macrophages and neutrophils, and at the dose of 3 mg/kg, it reduced the number of lymphocytes.

OVA-induced accumulation of inflammatory cells around the bronchioles and small vessels was detected in H&E stained paraffin-embedded lung sections (Figure 3A). As compared with vehicle, fisetin reduced this accumulation. Furthermore, fisetin also suppressed the OVA-induced goblet cell hyperplasia observed in paraffin-embedded sections stained with PAS (Figure 3B). Taken together, our data demonstrate that fisetin has anti-inflammatory effects in lungs from mice responding to OVA challenge.

Fisetin Reduced OVA-Induced Th2 Cytokines and Asthma-Related Chemokines. Inflammation in asthma is considered as a Th2 predominant immune reaction. To determine whether fisetin suppresses Th2 type inflammation in the lung, we measured the effects of fisetin on Th2 cytokine secretion, that is, on OVA-induced expression of hallmark Th2 cytokines, IL-4 and IL-5, in BAL fluid and lung tissues (Figure 4A,B). Notably, fisetin but not vehicle alone effectively decreased IL-4 and IL-5 in BAL fluid and lung parenchyma, suppressed the expression of IL-13 in lung parenchyma (Figure 4C), but had no effect on a key Th1 cytokine, interferon-γ (IFN-γ) (Figure 4A,B). Our data indicate that fisetin suppresses Th2 responses without affecting Th1 responses.

OVA inhalation also activated proinflammatory signals in bronchial epithelial cells causing them to secrete eotaxin-1 and TSLP. The mRNA expressions of both *eotaxin-1* and *TSLP* in lung parenchyma were much higher in OVA-challenged mice than PBS control group, and their expressions were significantly suppressed by fisetin (Figure 4C). Our data suggested that fisetin suppresses not only Th2 reactions but also proinflammatory signals associated with bronchial epithelial cells.

Fisetin Suppressed OVA-Induced Th2 Regulators in Cultured TLN Cells and Splenocytes. To investigate whether fisetin treatment directly suppress Th2-related responses in immune cells, we cultured TLN cells and splenocytes isolated from the mice under various treatments. IL-4 and IL-5 levels secreted by TLN cells and splenocytes were both higher from OVA-challenged mice than from control mice (Figure 5A,B), and fisetin reduced this OVA-induced IL-4 and IL-5 production.

Transcription factor GATA-3 plays essential roles in the transcriptional regulation of IL-4 and IL-5. The mRNA expression of *GATA-3* in TLN cells and splenocytes from OVA-challenged mice was higher than that from PBS-challenged mice (Figure 5C), and its expression was reduced in fisetin-treated mice. Taken together, these data suggest that fisetin inhibits the secretion of IL-4 and IL-5 in immune cells via suppressing the expression of GATA-3.

Fisetin Suppressed OVA-Induced AHR. Because fisetin suppressed Th2-related inflammation and goblet cell hyperplasia after OVA challenge, we then investigated whether fisetin attenuates AHR. We measured Penh using whole body plethysomograph in free-moving mice after exposure to increasing doses of methacholine, an airway constrictor (Figure 6). The degree of Penh increase was notably higher in OVA-challenged mice, and fisetin suppressed this increase.

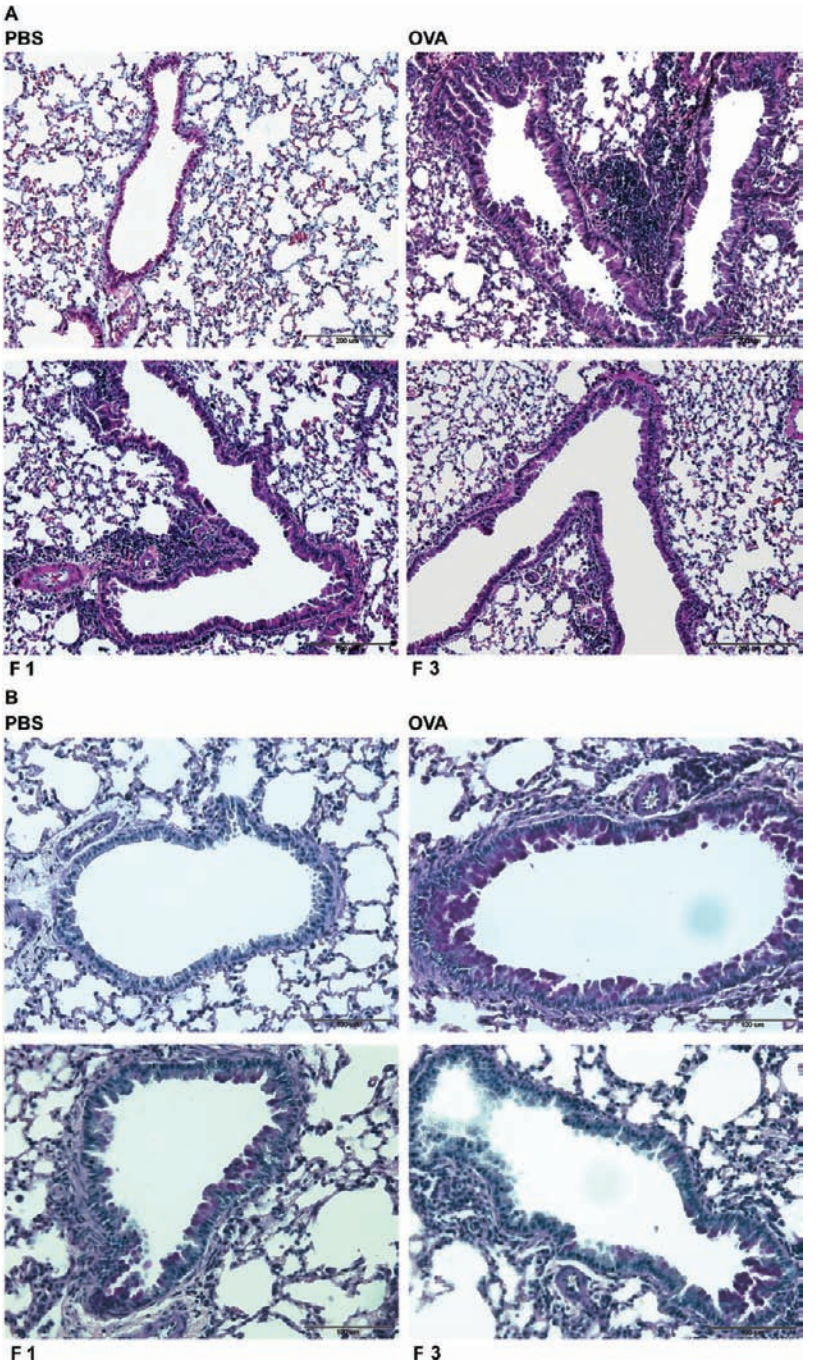

Figure 3. Fisetin suppressed OVA-induced lung inflammation and goblet cell hyperplasia. Lung tissues from mice subjected to various treatments were fixed, sectioned, and stained with (A) hematoxylin and eosin for microscopic examination (magnification, 100×) and (B) PAS with hematoxylin (magnification, 200×).

Fisetin Inhibited NF-κB Activation in Lung Tissues and Bronchial Epithelial Cells. To determine whether the therapeutic effect of fisetin on OVA-induced asthma is through NF-κB inhibition, we examined the expression of phosphorylated IκB-α and the nuclear localization of p65 subunit of NF-κB in lung tissues of mice subjected to OVA challenge. Fisetin suppressed phosphorylation of IκB-α in lung parenchyma (Figure 7A) and attenuated nuclear localization of p65 in lung tissues (Figure 7B).

We further investigated whether fisetin inhibited NF-κB activation in the bronchial epithelial cell line NL 20 using reporter assays. As shown in Figure 7C, fisetin (10 μM) inhibited TNF-α-induced NF-κB activation. Notably, fisetin treatment (10 μM, 24 h) had no cytotoxic effect on NL 20 cells (data not shown). These data demonstrated that fisetin could attenuate OVA-induced asthma through inhibition of NF-κB in lung tissues, and it could also suppress NF-κB activity in TNF-α-stimulated bronchial epithelial cells.

DISCUSSION

Our results revealed that fisetin attenuated OVA-induced lung inflammation, goblet cells hyperplasia, and AHR (Figures 3 and 6).

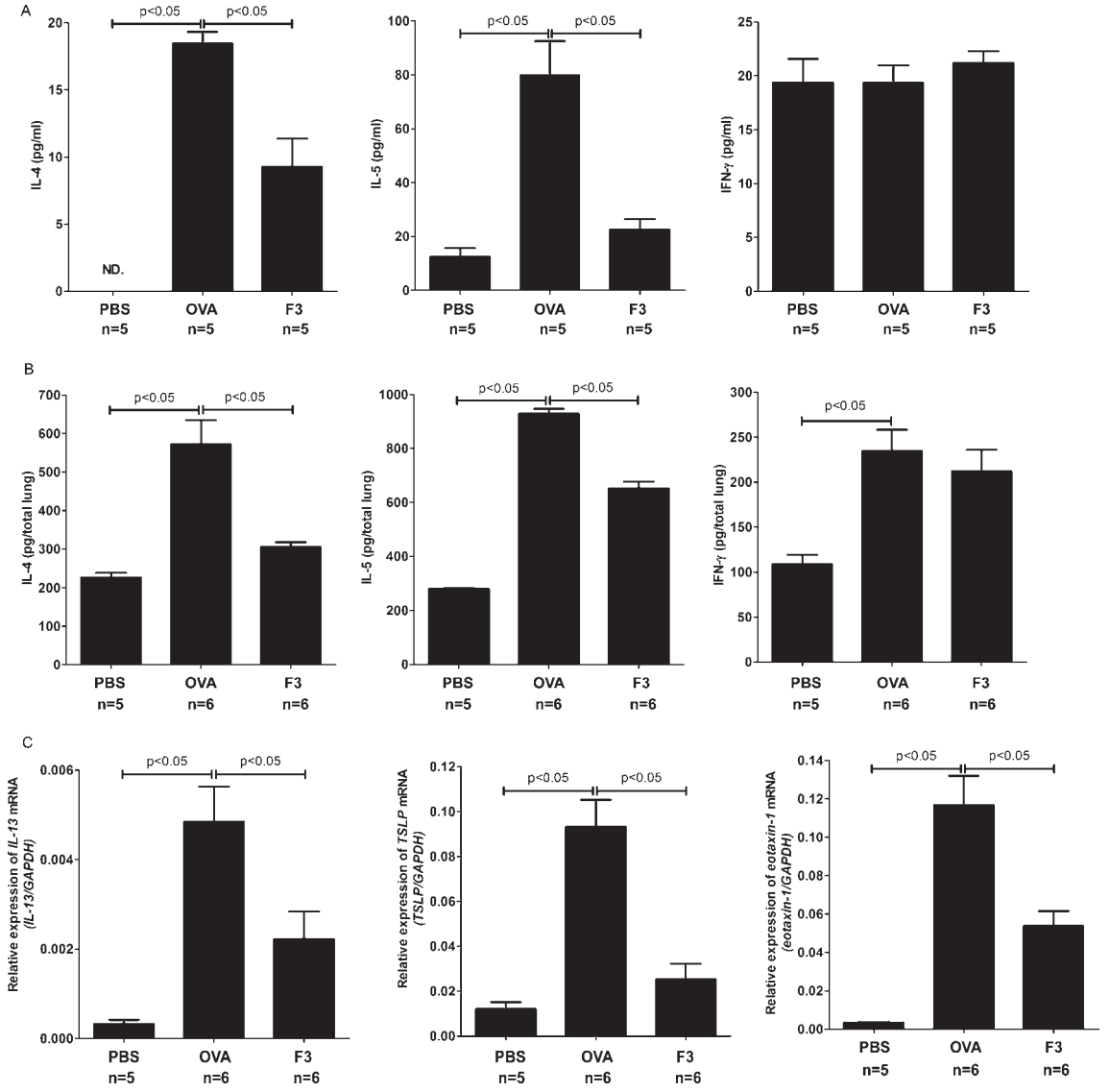

Figure 4. Fisetin reduced levels of Th2 cytokines and asthma-related chemokines in BAL fluid and lung parenchyma. BAL fluid and lung tissues were collected from mice subjected to various treatments 24 h after the final challenge. Cytokine levels in (A) BAL fluid and (B) total lung homogenates were determined by ELISA. (C) The expressions of indicated genes in lung tissues were determined by qRT-PCR. Values are shown as means ± SEMs, and $p < 0.05$ indicates significant difference.

Fisetin significantly reduced the numbers of infiltrating leukocytes in the airways of OVA-challenged mice, especially eosinophils and lymphocytes (Figure 2). Accordingly, OVA-induced Th2-associated cytokines (IL-4, IL-5, and IL-13) and transcription factor, GATA-3, were significantly suppressed by fisetin treatment (Figures 4 and 5). Eotaxin-1 and TSLP, secreted by bronchial epithelial cells, were also significantly suppressed by fisetin treatment (Figure 4). Fisetin also inhibited NF-κB activation in lung tissues and bronchial epithelial cells (Figure 7). Collectively, our data demonstrated that fisetin inhibited the Th2-dominant inflammation and AHR in OVA-induced asthma, revealing the beneficial effect of this compound for attenuating asthmatic responses.

Fisetin was identified as a NF-κB inhibitor because of its inhibitory effects on TAK-1 and IKK.[4] NF-κB is involved in the gene expressions of asthma-associated proinflammatory cytokines and chemokines (including TSLP and eotaxin-1[20,25]) and of GATA-3.[26] Our results showed expression of eotaxin-1 and TSLP in lung tissues, and GATA-3 in cultured TLN cells and splenocytes was reduced by fisetin and suggested that this reduction might be the consequence of NF-κB inhibition. Indeed, activation of NF-κB in bronchial epithelial cells and immune cells is highly associated with the development of asthma,[27,28] and several NF-κB-targeting strategies, such as small-molecule inhibitors and antisense oligonucleotides, are effective in experimental asthma models.[29−31] Fisetin was previously shown to suppress NF-κB activity in immune cells.[1] In this study, we showed fisetin could inhibit NF-κB activity in stimulated bronchial epithelial cells. Notably, phosphorylation of IκB-α and nuclear translocation of p65 in lung tissues were reduced upon fisetin treatment. Collectively, we hypothesized that fisetin suppressed OVA-induced asthma by inhibiting NF-κB activity in both epithelial cells and pro-inflammatory cells.

Jung et al. demonstrated that flavonols (including fisetin) attenuate acute asthmatic responses in guinea pig.[13] They found,

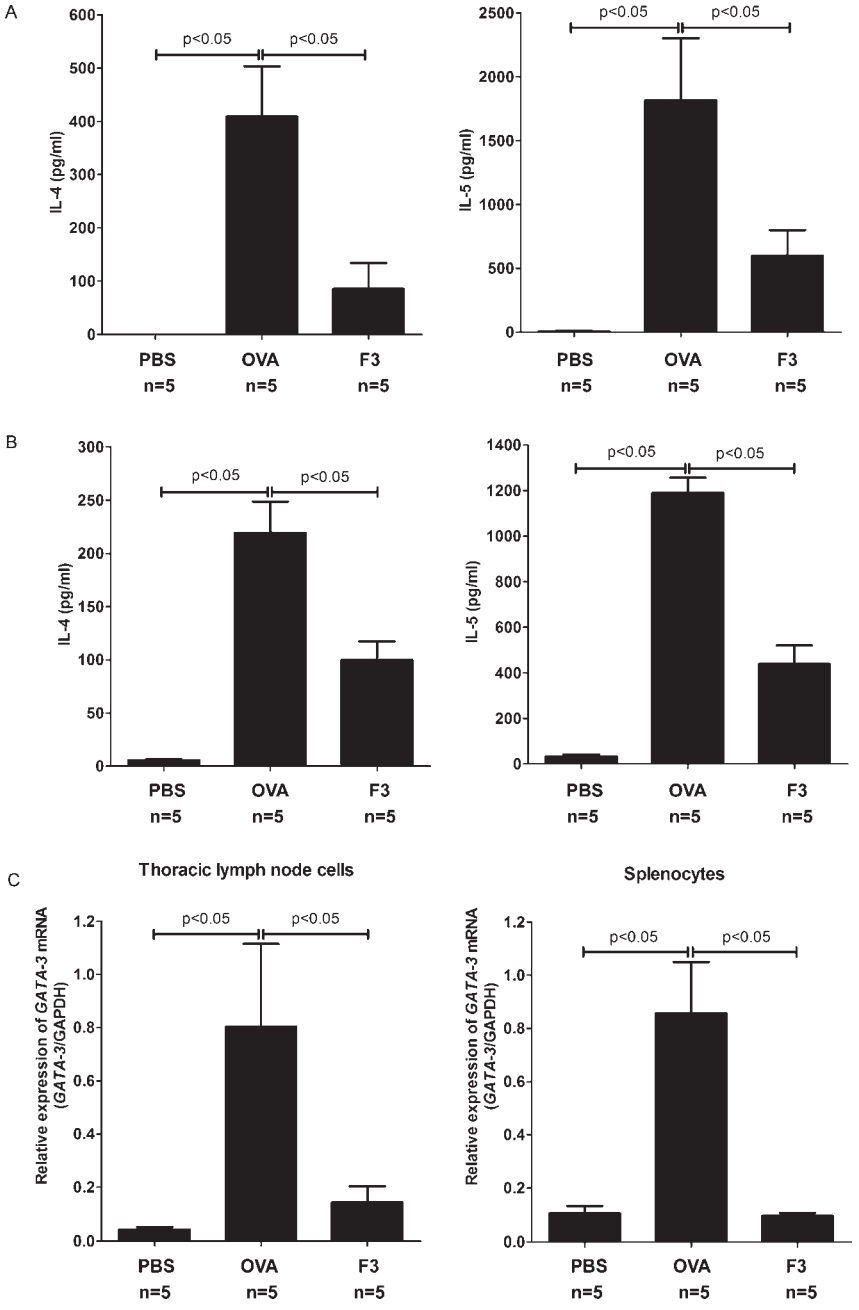

Figure 5. Fisetin inhibited the expression of the cytokines (IL-4 and IL-5) and transcription factor (GATA-3) in cultured TLN cells and splenocytes under OVA stimulation. Twenty-four hours after the final OVA challenge, isolated TLN cells and splenocytes were cultured in the presence of OVA (200 μg/mL) for 96 h. Culture media from (A) TLN cells and (B) splenocytes of mice treated under the indicated conditions were collected, and the levels of IL-4 and IL-5 were measured using ELISA. (C) The mRNA expression of *GATA-3* in TLN cells and splenocytes was measured by qRT-PCR. Values are shown as means ± SEMs, and $p < 0.05$ means significant difference.

as did we, that fisetin could inhibit recruitment of eosinophils. However, in their study, fisetin suppressed histamine, erythropoietin (EPO), and phospholipase A_2 (PLA_2) activity in BAL fluid, which only reflected the inhibitory effects of fisetin on the activation of mast cells, eosinophils, and neutrophils. The effects of fisetin on Th2 reactions, the predominant immune responses in asthma, were not addressed. Our study demonstrated, for the first time, that fisetin downregulated Th2-associated regulators (GATA-3, IL-4, IL-5, and IL-13) as well as bronchial epithelial cell-related cytokine and chemokine (TSLP and eotaxin-1) in a murine asthma model. Moreover, fisetin inhibited NF-κB in lung tissues and cultured bronchial epithelial cells. Our results further elucidate the molecular mechanisms underlying the antiasthmatic effect of fisetin.

In the immunopathogenesis of asthma, the main antigen presenting cells (APCs) for activating Th2 cells are dendritic cells. However, recent data indicate that eosinophils not only can function as effector cells but also can act as APC for activating and recruiting Th2 cells in asthma.[32] Therefore, the eosinophil is a major contributor to the pathogenesis of allergic asthma. Notably, our study showed that fisetin mainly reduced eosinophil counts in BAL fluid, suggesting that the eosinophil is the main cell target of fisetin. In the process of eosinophil accumulation, IL-5 induces the release of

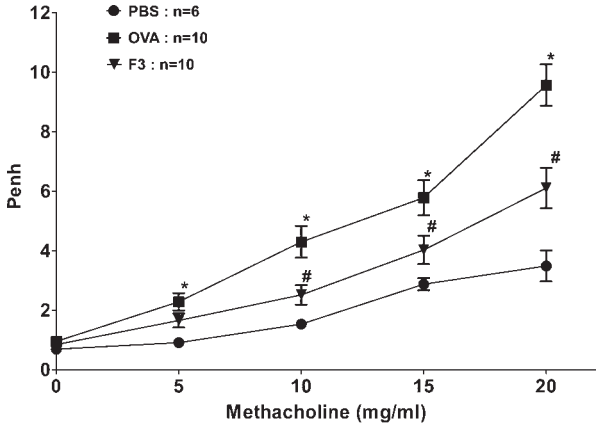

Figure 6. Fisetin suppressed OVA-induced AHR. AHR was determined using whole body plethysmograph 24 h after the final challenge. Values are shown as means ± SEMs; * indicates significant difference vs PBS group ($p < 0.05$). # means significant difference vs OVA group ($p < 0.05$).

eosinophils from bone marrow to blood, and eotaxin-1 and IL-13 induce the transfer of eosinophils from blood to target tissues.[33] Recent evidence also demonstrated that TSLP activate eosinophils.[34] On the basis of our results showing that fisetin suppressed eosinophil accumulation in BAL fluid and the expressions of IL-5, IL-13, eotaxin-1, and TSLP, we speculated that fisetin treatment impaired the recruitment and activation of eosinophils via suppressing the expressions of these cytokines and chemokines. Certainly, the exact mechanisms by which fisetin affects eosinophils merit further investigation.

In this study, ip injection was used to demonstrate the antiasthma activity of fisetin in vivo. The dosing regimen used in this study is based on our previous study showing in vivo immunosuppressive effect of fisetin.[1] Recently, a study by Shia et al. describes the pharmacokinetic profiles of fisetin via oral (50 mg/kg; single injection) and intravenous (10 mg/kg; single injection) administration in rats.[35] Their data indicate that fisetin po is more rapidly metabolized than fisetin iv. Because fisetin via iv or ip administration does not pass through the gastrointestinal system, we assume the pharmacokinetic profile of fisetin ip will be more similar to that of fisetin iv. Nevertheless, we carried out multiple ip injections in this study (Figure 1), which presumably causes more complicated pharmacokinetic profile than that of single injection. Thus, the actual pharmacokinetic profiles of fisetin in our study remained to be further determined.

Several immunosuppressive drugs, such as corticosteroids, β_2-adrenergic agonist, and cytokines (TGF-β and IL-10), are available for treating allergic diseases,[36] but most of them have significant side effects. Recent experimental evidence from in vitro and in vivo studies supports that dietary flavonoid compounds (such as fisetin, quercetin, and luteolin) have potent anti-inflammatory activities.[13,37] Furthermore, epidemiological studies reported that a low incidence of asthma was found in individuals who intake high amount of flavonoids.[38] Our study has demonstrated that fisetin effectively attenuate asthmatic phenotypes, supporting that it is a promising candidate compound for alleviating allergic diseases. Because fisetin is commonly present in dietary plants and the fisetin dosages used in our study did not appear to cause any deleterious side effects, this compound presumably is safer than current immunosuppressive drugs. Certainly, the molecular basis and clinical implications of fisetin-mediated antiasthmatic activity merit further investigation.

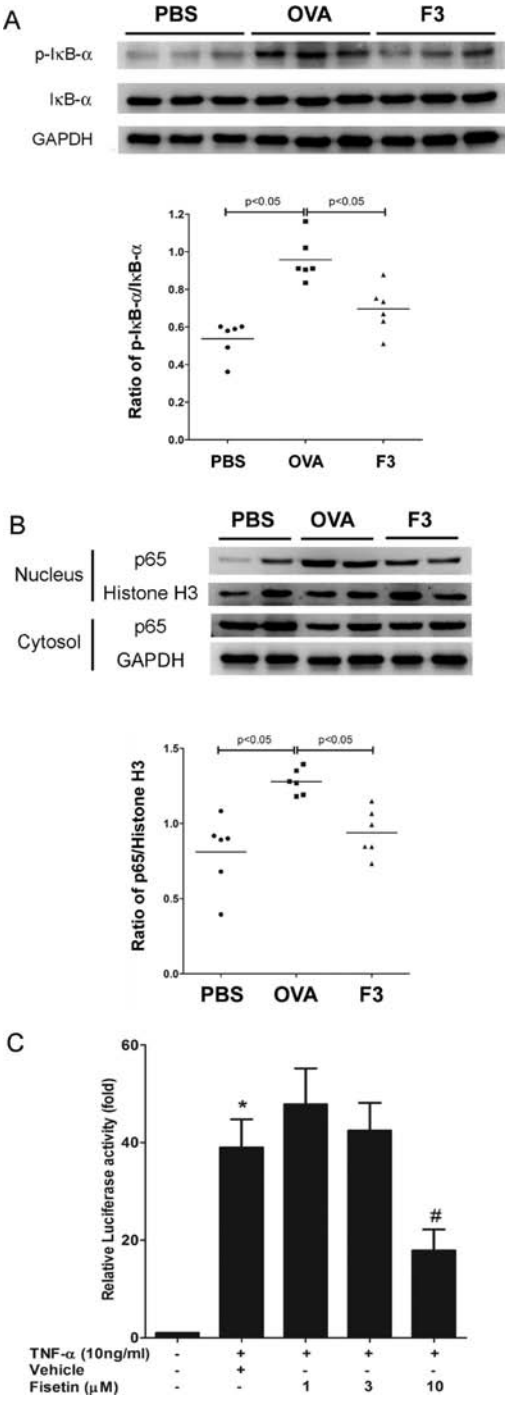

Figure 7. Fisetin suppressed OVA-induced IκB-α phosphorylation and p65 translocation in lung tissues and attenuated TNF-α-induced NF-κB activation in bronchial epithelial cells. (A) Phosphorylated IκB-α (p-IκB-α) and total IκB-α in cytosolic lysates of lung tissues (50 μg per lane) were detected by Western blot, where GAPDH was the internal control. Quantification data ($n = 6$ for each treatment group) are shown in the bottom panel. (B) Immunoblotting of p65 in cytosolic and nuclear extracts of lung tissues (50 μg per lane) were carried out, where histone H3 and GAPDH were the internal controls. Quantification data ($n = 6$ for each treatment group) were shown in bottom panel. (C) Relative luciferase activities in NL 20 cells under various treatments. * indicates significant difference vs control ($p < 0.05$). # means significant difference vs vehicle ($p < 0.05$).

AUTHOR INFORMATION

Corresponding Author

*Tel: 886-2-28267177. Fax: 886-2-28225044. E-mail: slfu@ym.edu.tw.

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REFERENCES

(1) Liu, S. H.; Lin, C. H.; Hung, S. K.; Chou, J. H.; Chi, C. W.; Fu, S. L. Fisetin inhibits lipopolysaccharide-induced macrophage activation and dendritic cell maturation. *J. Agric. Food Chem.* **2010**, *58* (20), 10831–10839.

(2) Arai, Y.; Watanabe, S.; Kimira, M.; Shimoi, K.; Mochizuki, R.; Kinae, N. Dietary intakes of flavonols, flavones and isoflavones by Japanese women and the inverse correlation between quercetin intake and plasma LDL cholesterol concentration. *J. Nutr.* **2000**, *130* (9), 2243–2250.

(3) Hanneken, A.; Lin, F. F.; Johnson, J.; Maher, P. Flavonoids protect human retinal pigment epithelial cells from oxidative-stress-induced death. *Invest. Ophthalmol. Vis. Sci.* **2006**, *47* (7), 3164–3177.

(4) Sung, B.; Pandey, M. K.; Aggarwal, B. B. Fisetin, an inhibitor of cyclin-dependent kinase 6, down-regulates nuclear factor-kappaB-regulated cell proliferation, antiapoptotic and metastatic gene products through the suppression of TAK-1 and receptor-interacting protein-regulated IkappaBalpha kinase activation. *Mol. Pharmacol.* **2007**, *71* (6), 1703–1714.

(5) Fotsis, T.; Pepper, M. S.; Montesano, R.; Aktas, E.; Breit, S.; Schweigerer, L.; Rasku, S.; Wahala, K.; Adlercreutz, H. Phytoestrogens and inhibition of angiogenesis. *Baillieres Clin. Endocrinol. Metab.* **1998**, *12* (4), 649–666.

(6) Higa, S.; Hirano, T.; Kotani, M.; Matsumoto, M.; Fujita, A.; Suemura, M.; Kawase, I.; Tanaka, T. Fisetin, a flavonol, inhibits TH2-type cytokine production by activated human basophils. *J. Allergy Clin. Immunol.* **2003**, *111* (6), 1299–1306.

(7) Park, H. H.; Lee, S.; Son, H. Y.; Park, S. B.; Kim, M. S.; Choi, E. J.; Singh, T. S.; Ha, J. H.; Lee, M. G.; Kim, J. E.; Hyun, M. C.; Kwon, T. K.; Kim, Y. H.; Kim, S. H. Flavonoids inhibit histamine release and expression of proinflammatory cytokines in mast cells. *Arch. Pharm. Res.* **2008**, *31* (10), 1303–1311.

(8) Park, H. H.; Lee, S.; Oh, J. M.; Lee, M. S.; Yoon, K. H.; Park, B. H.; Kim, J. W.; Song, H.; Kim, S. H. Anti-inflammatory activity of fisetin in human mast cells (HMC-1). *Pharmacol. Res.* **2007**, *55* (1), 31–37.

(9) Wang, L.; Tu, Y. C.; Lian, T. W.; Hung, J. T.; Yen, J. H.; Wu, M. J. Distinctive antioxidant and antiinflammatory effects of flavonols. *J. Agric. Food Chem.* **2006**, *54* (26), 9798–9804.

(10) Lyu, S. Y.; Park, W. B. Production of cytokine and NO by RAW 264.7 macrophages and PBMC in vitro incubation with flavonoids. *Arch. Pharm. Res.* **2005**, *28* (5), 573–581.

(11) Geraets, L.; Haegens, A.; Brauers, K.; Haydock, J. A.; Vernooy, J. H.; Wouters, E. F.; Bast, A.; Hageman, G. J. Inhibition of LPS-induced pulmonary inflammation by specific flavonoids. *Biochem. Biophys. Res. Commun.* **2009**, *382* (3), 598–603.

(12) Lee, J. D.; Huh, J. E.; Jeon, G.; Yang, H. R.; Woo, H. S.; Choi, D. Y.; Park, D. S. Flavonol-rich RVHxR from Rhus verniciflua Stokes and its major compound fisetin inhibits inflammation-related cytokines and angiogenic factor in rheumatoid arthritic fibroblast-like synovial cells and in vivo models. *Int. Immunopharmacol.* **2009**, *9* (3), 268–276.

(13) Jung, C. H.; Lee, J. Y.; Park, J. H.; Cho, B. J.; Sim, S. S.; Kim, C. J. Flavonols attenuate the immediate and late-phase asthmatic responses to aerosolized-ovalbumin exposure in the conscious guinea pig. *Fitoterapia* **2010**, *81* (7), 803–812.

(14) Hamid, Q.; Tulic, M. Immunobiology of asthma. *Annu. Rev. Physiol.* **2009**, *71*, 489–507.

(15) Barrett, N. A.; Austen, K. F. Innate cells and T helper 2 cell immunity in airway inflammation. *Immunity* **2009**, *31* (3), 425–437.

(16) Takatsu, K.; Nakajima, H. IL-5 and eosinophilia. *Curr. Opin. Immunol.* **2008**, *20* (3), 288–294.

(17) Walsh, E. R.; August, A. Eosinophils and allergic airway disease: There is more to the story. *Trends Immunol.* **2010**, *31* (1), 39–44.

(18) Kuperman, D. A.; Huang, X.; Koth, L. L.; Chang, G. H.; Dolganov, G. M.; Zhu, Z.; Elias, J. A.; Sheppard, D.; Erle, D. J. Direct effects of interleukin-13 on epithelial cells cause airway hyperreactivity and mucus overproduction in asthma. *Nat. Med.* **2002**, *8* (8), 885–889.

(19) Ziegler, S. F.; Artis, D. Sensing the outside world: TSLP regulates barrier immunity. *Nat. Immunol.* **2010**, *11* (4), 289–293.

(20) Lee, H. C.; Ziegler, S. F. Inducible expression of the proallergic cytokine thymic stromal lymphopoietin in airway epithelial cells is controlled by NF-kappaB. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104* (3), 914–919.

(21) Hart, L. A.; Krishnan, V. L.; Adcock, I. M.; Barnes, P. J.; Chung, K. F. Activation and localization of transcription factor, nuclear factor-kappaB, in asthma. *Am. J. Respir. Crit. Care Med.* **1998**, *158* (5 Part 1), 1585–1592.

(22) Desmet, C.; Gosset, P.; Pajak, B.; Cataldo, D.; Bentires-Alj, M.; Lekeux, P.; Bureau, F. Selective blockade of NF-kappa B activity in airway immune cells inhibits the effector phase of experimental asthma. *J. Immunol.* **2004**, *173* (9), 5766–5775.

(23) Kim, D. Y.; Ryu, S. Y.; Lim, J. E.; Lee, Y. S.; Ro, J. Y. Anti-inflammatory mechanism of simvastatin in mouse allergic asthma model. *Eur. J. Pharmacol.* **2007**, *557* (1), 76–86.

(24) Hung, L. C.; Lin, C. C.; Hung, S. K.; Wu, B. C.; Jan, M. D.; Liou, S. H.; Fu, S. L. A synthetic analog of alpha-galactosylceramide induces macrophage activation via the TLR4-signaling pathways. *Biochem. Pharmacol.* **2007**, *73* (12), 1957–1970.

(25) Yang, L.; Cohn, L.; Zhang, D. H.; Homer, R.; Ray, A.; Ray, P. Essential role of nuclear factor-kappaB in the induction of eosinophilia in allergic airway inflammation. *J. Exp. Med.* **1998**, *188* (9), 1739–1750.

(26) Das, J.; Chen, C. H.; Yang, L.; Cohn, L.; Ray, P.; Ray, A. A critical role for NF-kappa B in GATA3 expression and TH2 differentiation in allergic airway inflammation. *Nat. Immunol.* **2001**, *2* (1), 45–50.

(27) Pantano, C.; Ather, J. L.; Alcorn, J. F.; Poynter, M. E.; Brown, A. L.; Guala, A. S.; Beuschel, S. L.; Allen, G. B.; Whittaker, L. A.; Bevelander, M.; Irvin, C. G.; Janssen-Heininger, Y. M. Nuclear factor-kappaB activation in airway epithelium induces inflammation and hyperresponsiveness. *Am. J. Respir. Crit. Care Med.* **2008**, *177* (9), 959–969.

(28) Gagliardo, R.; Chanez, P.; Mathieu, M.; Bruno, A.; Costanzo, G.; Gougat, C.; Vachier, I.; Bousquet, J.; Bonsignore, G.; Vignola, A. M. Persistent activation of nuclear factor-kappaB signaling pathway in severe uncontrolled asthma. *Am. J. Respir. Crit. Care Med.* **2003**, *168* (10), 1190–1198.

(29) Choi, I. W.; Kim, D. K.; Ko, H. M.; Lee, H. K. Administration of antisense phosphorothioate oligonucleotide to the p65 subunit of NF-kappaB inhibits established asthmatic reaction in mice. *Int. Immunopharmacol.* **2004**, *4* (14), 1817–1828.

(30) Birrell, M. A.; Hardaker, E.; Wong, S.; McCluskie, K.; Catley, M.; De Alba, J.; Newton, R.; Haj-Yahia, S.; Pun, K. T.; Watts, C. J.; Shaw, R. J.; Savage, T. J.; Belvisi, M. G. Ikappa-B kinase-2 inhibitor blocks inflammation in human airway smooth muscle and a rat model of asthma. *Am. J. Respir. Crit. Care Med.* **2005**, *172* (8), 962–971.

(31) Bao, Z.; Guan, S.; Cheng, C.; Wu, S.; Wong, S. H.; Kemeny, D. M.; Leung, B. P.; Wong, W. S. A novel antiinflammatory role for

andrographolide in asthma via inhibition of the nuclear factor-kappaB pathway. *Am. J. Respir. Crit. Care Med.* **2009**, *179* (8), 657–665.

(32) Jacobsen, E. A.; Ochkur, S. I.; Pero, R. S.; Taranova, A. G.; Protheroe, C. A.; Colbert, D. C.; Lee, N. A.; Lee, J. J. Allergic pulmonary inflammation in mice is dependent on eosinophil-induced recruitment of effector T cells. *J. Exp. Med.* **2008**, *205* (3), 699–710.

(33) Humbles, A. A.; Conroy, D. M.; Marleau, S.; Rankin, S. M.; Palframan, R. T.; Proudfoot, A. E.; Wells, T. N.; Li, D.; Jeffery, P. K.; Griffiths-Johnson, D. A.; Williams, T. J.; Jose, P. J. Kinetics of eotaxin generation and its relationship to eosinophil accumulation in allergic airways disease: Analysis in a guinea pig model in vivo. *J. Exp. Med.* **1997**, *186* (4), 601–612.

(34) Wong, C. K.; Hu, S.; Cheung, P. F.; Lam, C. W. TSLP Induces Chemotactic and Pro-survival Effects in Eosinophils: Implications in Allergic Inflammation. *Am. J. Respir. Cell Mol. Biol.* **2010**, *43* (3), 305–315.

(35) Shia, C. S.; Tsai, S. Y.; Kuo, S. C.; Hou, Y. C.; Chao, P. D. Metabolism and pharmacokinetics of 3,3′,4′,7-tetrahydroxyflavone (fisetin), 5-hydroxyflavone, and 7-hydroxyflavone and antihemolysis effects of fisetin and its serum metabolites. *J. Agric. Food Chem.* **2009**, *57* (1), 83–89.

(36) Adcock, I. M.; Caramori, G.; Chung, K. F. New targets for drug development in asthma. *Lancet* **2008**, *372* (9643), 1073–1087.

(37) Kawai, M.; Hirano, T.; Higa, S.; Arimitsu, J.; Maruta, M.; Kuwahara, Y.; Ohkawara, T.; Hagihara, K.; Yamadori, T.; Shima, Y.; Ogata, A.; Kawase, I.; Tanaka, T. Flavonoids and related compounds as anti-allergic substances. *Allergol. Int.* **2007**, *56* (2), 113–123.

(38) Serafini, M.; Peluso, I.; Raquzzini, A. Flavonoids as anti-inflammatory agents. *Proc. Nutr. Soc.* **2010**, *69* (3), 273–278.